



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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<b>(21) International Application Number:</b> PCT/EP98/01973 <b>(22) International Filing Date:</b> 3 April 1998 (03.04.98) <b>(30) Priority Data:</b> PD97A000064      4 April 1997 (04.04.97)      IT PD98A000022      10 February 1998 (10.02.98)      IT <b>(71) Applicant (for all designated States except US):</b> FIDIA ADVANCED BIOPOLYMERS, S.R.L. [IT/IT]; Via De Carpentieri, 3, I-72100 Brindisi (IT). <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only):</b> RENIER, David [IT/IT]; Via degli Alpini, 4, I-35035 Mestrino Padue (IT). CALLEGARO, Lanfranco [IT/IT]; Via Monte Grappa, 6, I-36016 Thiene Vicenza (IT). <b>(74) Agent:</b> VOSSIUS & PARTNER; Siebertstrasse 4, D-81675 München (DE).		<b>(81) Designated States:</b> AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, GW, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
<b>(54) Title:</b> N-SULPHATED HYALURONIC ACID COMPOUNDS, DERIVATIVES THEREOF AND A PROCESS FOR THEIR PREPARATION		
<b>(57) Abstract</b>  <p>The present invention is directed to novel sulphated compounds of hyaluronic acid and derivatives thereof, optionally salified, wherein the glucosamines are partially N-sulphated or partially N-sulphated and partially or totally O-sulphated in position 6. The compounds of the invention have anticoagulant and antithrombotic activities and are useful in the preparation of pharmaceutical compositions and biomaterials and in the production of coatings for biomedical objects.</p>		

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N-SULPHATED HYALURONIC ACID COMPOUNDS,  
DERIVATIVES THEREOF  
AND A PROCESS FOR THEIR PREPARATION

FIELD OF THE INVENTION

The present invention concerns new sulphated compounds of hyaluronic acid and derivatives thereof having anticoagulant and antithrombotic activities and processes for their preparation. Said compounds are useful in the preparation of pharmaceutical compositions and biomaterials and in the production of coatings for biomedical objects.

BACKGROUND OF THE INVENTION

Heparin is the sulphated glycosaminoglycan with the greatest biological activity. Its antithrombotic and anticoagulant properties are well known. Indeed, it is used in treatment for cardiovascular pathologies where there is a risk of thrombosis, and it has contributed notably to the successful outcome of open-heart surgery. The structure of heparin is not altogether known.

Commercial heparin comprises a range of 21 different kinds (Nader et al., 1974, Biochem. Biophys. Res. Commun. 57:488), with a molecular weight of between 3,000 and 37,500 Da, and with varying anticoagulant activity.

Heparin's anticoagulant activity depends on its structural characteristics, for example, on the degree of sulphation, on the degree of dissociation, on the sequence of the COO<sup>-</sup> and SO<sub>3</sub><sup>-</sup> groups, on the

shape and size of the molecule. These factors are important to the formation of the ion bonds responsible for heparin's biological activity (Stivala et al., 1967, Arch. Biochem. Biophys. 122:40).

Because of the high density of its negative charge, heparin has a strong affinity for cations and its activity is pH-dependent. In particular, the N-sulphated group of its glucosamine residue plays a fundamental role in the interaction with the factors regulating the coagulative processes.

A significant reduction in the N-sulphated groups drastically reduces its anticoagulant and antithrombotic activities.

Many natural polysaccharides have been sulphated in order to obtain heparin-like products (Hoffman et al., 1982, Carbohydrate Res. 2:115; Kindness et al., 1980, Brit. J. Pharmac. 69:675; Horton et al., 1973, Carbohydrate Res. 30:349; Okada et al., 1979, Makromol. Chem. 180:813; Kikuchi et al., 1979, Nippon Kagaku Kaishi 1:127; Manzac et al., 1981, Proc. Third M.I.S.A.O. 5:504). Moreover, sulphuric, carboxy or sulphonated groups have been attached to synthetic polymers such as polystyrene (Kanmaugue et al., 1985, Biomaterials, 6:297) and polyurethanes (Ito et al., 1992, Biomaterials, 13:131).

However, the anticoagulant activity of these materials is much lower than that of heparin and depends on the type of substituent, the type of bond, the degree of substitution and the sequence.

Lastly, some chemical reactions for the sulphation of polysaccharides are known (WO 88/00211; EP 0340628; Carbohydrate Research, 158, 183-190, 1986) but no derivatives have ever been obtained which present, besides the chemical-physical characteristics peculiar to polysaccharides, any new characteristics such as anticoagulant activity.

In the international patent application, Publication No. WO 95/25751, a process is described for the non-selective sulphation of

hyaluronic acid and the derivatives thereof to obtain compounds with an antithrombotic activity.

5 Their ability to inactivate thrombin is due to the formation of electrostatic interactions depending on the charge density, which increases according to the degree of sulphation, while heparin's activity is the consequence of a direct interaction with antithrombin III (T.W. Barrowcliffe et al., Journal of Pharmaceutical & Biomedical Analysis, Vol. 7, No. 2, pages 217-226, 1989; Peter D. J. Grootenhuis et al., J. Am. Chem. Soc., 113, 2743-2747, 1991).

10 Heparin is widely used, although it does present side effects such as haemorrhagic effects, which prevent its being used too freely or without medical guidance.

15 Moreover, because of its chemical-physical characteristics, heparin cannot be used as a biomaterial but simply as a coating for other materials, and in sufficiently small quantities to avoid its causing localized bleeding.

20 Lastly, by acting directly on the coagulation factors, the anticoagulant action of heparin begins very rapidly and its duration, albeit dose-dependent, is generally similarly brief. These drawbacks limit its applicability in certain surgical fields such as cardiovascular surgery involving the implantation of devices requiring an absolute absence of thrombogenicity for a given length of time.

#### SUMMARY OF THE INVENTION

25 The present invention is directed to novel sulphated compounds of hyaluronic acid and the derivatives thereof, optionally salified, with an anticoagulant and antithrombotic activity, wherein the glucosamines are partially N-sulphated or partially N-sulphated and partially or totally O-sulphated in position 6, for the preparation of pharmaceutical  
30 formulations, biocompatible and bioabsorbable biomaterials with an

anticoagulant activity and for the coating of biomedical objects and processes for their preparation.

The present invention also provides for a chemical process using a well-characterized starting product such as hyaluronic acid, which process allows for the selective sulphation of the amino group of glucosamine or the hydroxy group in the position 6, to thus obtain new sulphated derivatives of hyaluronic acid with an unaltered range of molecular weights and with an anticoagulant activity similar to that of heparin.

While the ability of hypersulphated polysaccharides to inactivate thrombin is due to the formation of electrostatic interactions depending on the charge density, which increases according to the degree of sulphation, the N-sulphated derivatives provided in the present invention appear to act on the coagulation factors by means of a specific mechanism similar to that of heparin.

Further advantages of the present invention are represented by the improved chemical-physical characteristics of the N-sulphated derivatives compared to those of the hyper-sulphated derivatives, making them suitable for the preparation of biomaterials for use in the fields of biomedicine and health care and in the pharmaceutical field.

Moreover, the possibility of obtaining a compound with an anticoagulant and non-thrombogenic activity by means of selective sulphation of just the amino groups and hydroxy groups in position 6, notably reduces the costs of the process compared to the preparation of hyaluronic acid with all the hydroxy groups homogeneously sulphated.

#### DETAILED DESCRIPTION OF THE INVENTION

The term "partially 2-N-sulphated derivative" of hyaluronic acid as used herein means a product obtained by means of a controlled sulphation reaction of the amino group of the glucosamine of hyaluronic

acid, previously N-deacetylated according to the procedure described by P. Shaklee (1984) Biochem. J. 217, 187-197. The reaction proceeds as illustrated below:

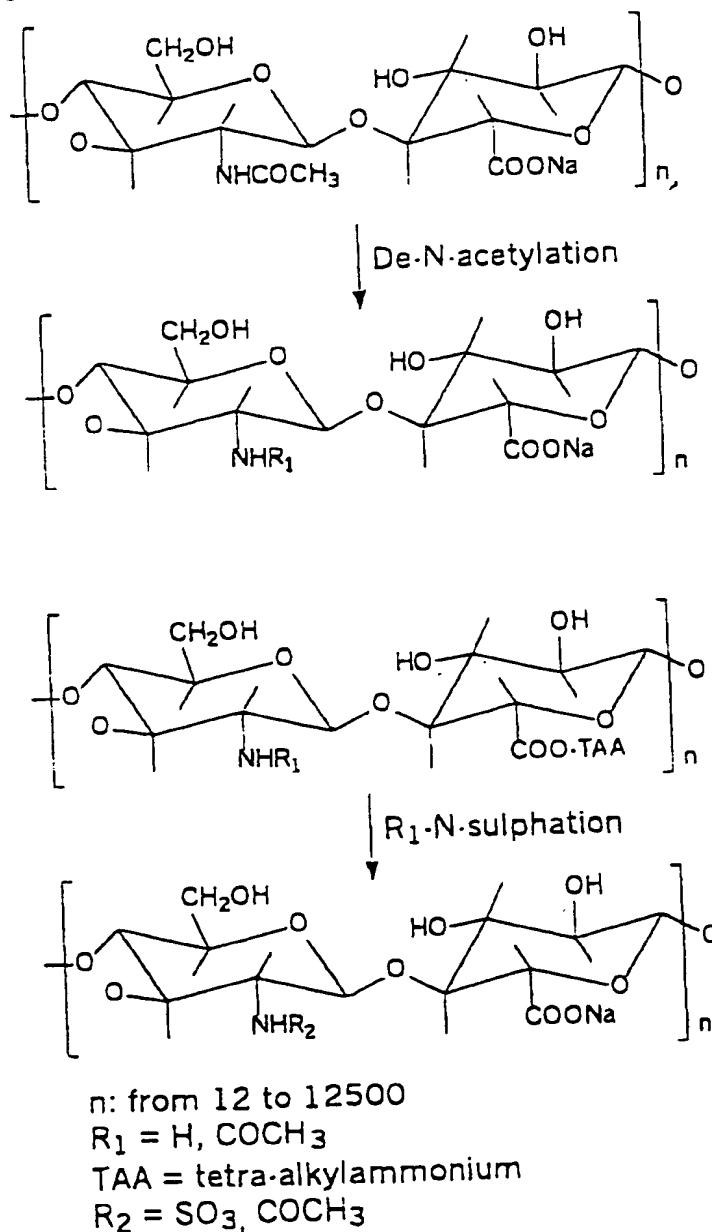
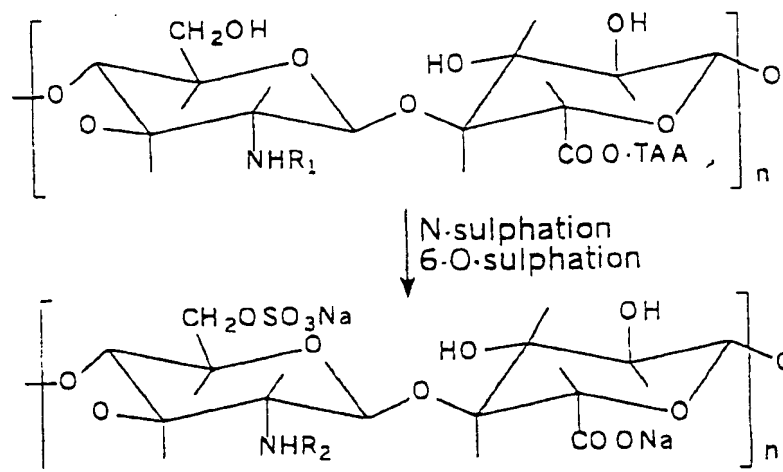


Diagram 1

The term "partially 2-N-sulphated and 6-O-sulphated derivatives" as used herein means the products of the chemical reaction illustrated

6

in diagram 1, wherein, besides the amino group of glucosamine, the primary hydroxy function of the same residue is also totally or partially involved in the sulphation reaction, as illustrated below:



n: from 12 to 12500

$R_1 = \text{H}, \text{COCH}_3$

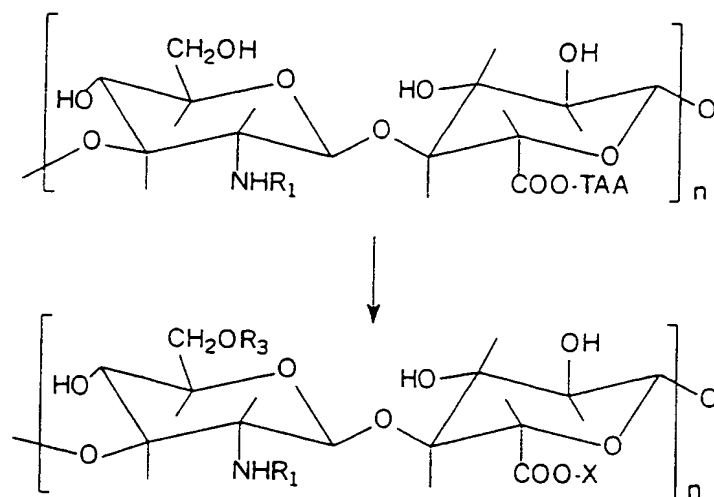
TAA = tetra-alkylammonium

$R_2 = \text{SO}_3, \text{COCH}_3$

Diagram 2

5 The derivatives generated according to diagrams 1 and 2 can be used as intermediate reactants in the preparation of compounds, according to the procedure described in European patent 0216453 B1, wherein the carboxy function of the glucuronic residue of hyaluronic acid, partially 2-N-sulphated or partially 2-N-sulphated and partially or  
10 totally 6-O-sulphated, is partially or completely reacted with alcohols of the aliphatic, aromatic, arylaliphatic, cycloaliphatic, heterocyclic series, producing the respective partial or total esters:





n: from 12 to 12500

R<sub>1</sub> = H, COCH<sub>3</sub>

TAA = tetra-alkylammonium

R<sub>2</sub> = SO<sub>3</sub>, COCH<sub>3</sub>

R<sub>3</sub> = SO<sub>3</sub>, H

X = alcoholic residue, Sodium

**Diagram 3**

Moreover, it is possible to use the synthetic derivatives according to diagrams 1 and 2 as intermediates in the preparation of crosslinked compounds, according to the procedures described in European patents 0341745 B1 and 265116 B1 respectively, wherein a part or all of the carboxy groups belonging to the D-glucuronic residue are reacted: i) using condensing agents with the alcohol functions of the same polysaccharide chain or other chains, generating inner (or lactone) esters and intermolecular esters; ii) with poly-alcohols of the aliphatic, aromatic, arylaliphatic, cycloaliphatic, heterocyclic series, generating crosslinking by means of spacer chains.

The above-said sulphated compounds obtained according to the process of the present invention can be optionally salified with heavy metals, the heavy metals being selected from the group of metal

elements in the 4th, 5th and 6th periods of the periodical table, such as silver, iron, cobalt, copper, zinc, arsenic, strontium, zirconium, antimonium, gold, cesium, tungsten, selenium, platinum, ruthenium, bismuth, tin, titanium, and mercury. These salts can be used in dermatology, ophthalmology, dentistry, stomatology, rheumatology, urology, gynecology, internal surgery, as food supplements and as anti-oxidant, antirheumatic, antitumoral, antiinflammatory, analgesic and anti-ulcer agents.

Lastly, the sulphated derivatives can be optionally salified with pharmacologically active substances such as antibiotics, antiinfective, antimicrobial, antiviral, cytostatic, antitumoral, antiinflammatory and wound healing agents, anesthetics, cholinergic or adrenergic agonists or antagonists, antithrombotic, anticoagulant, haemostatic, fibrinolytic and thrombolytic agents, proteins and their fragments, peptides, and polynucleotides.

Apart from their above noted antithrombotic and anticoagulant properties and their biocompatibility characteristics, the N-sulphated derivatives of the present invention can be used advantageously as antiviral agents, for example against human immune deficiency virus (HIV) and herpes, and as anti-inflammatories for systemic, topical or local use, for example by the rectal or vaginal routes both in the form of pharmaceutical compositions and in the form of biomaterials.

The present inventive compounds and their salts can therefore be used advantageously, either alone or in association with one another or with other pharmacologically active substances, in combination with a pharmaceutically acceptable carrier for the preparation of pharmaceutical compositions.

Such pharmaceutical compositions can be used, for example, as antithrombotic, anticoagulant, anti-inflammatory, antiviral, anti-oedematous preparations, to accelerate wound healing, in the treatment

of burns, sores, skin ulcers, dental decay, restenosis and infarction and to favor angiogenesis.

Of special interest are formulations and biomaterials for the transport and release of biologically active substances such as proteins and their fragments, peptides, polynucleotides, growth factors, enzymes, vaccines, substances used in the treatment of diseases associated with genetic defects such as those depending on enzymatic hypo- or hyperactivity due to defects of the gene encoding for a given enzyme, deforming diseases and hereditary disorders.

The sulphated derivatives according to the present invention can be associated with radioactive and non-radioactive substances used in contrast systems, and used as tracers in *in vivo* diagnostics in the identification and cure of tumoral or damaged tissues.

One considerable advantage is represented by the possibility of processing the sulphated compounds and their salts in various forms of biomaterials such as sponges, films, membranes, threads, tampons, nonwoven fabrics, microspheres, nanospheres, gauzes, gels, guide channels. These biomaterials, in one such form or several forms together, can be constituted by one or more sulphated derivatives and by their salts, optionally included in association with other natural, synthetic, semisynthetic polymers and, optionally, with biologically active substances.

Examples of the natural polymers which can be used are collagen, coprecipitates of collagen and glycosaminoglycans, cellulose, polysaccharides in the form of gels such as chitin, chitosan, pectin or pectic acid, agar, agarose, xanthan, gellan, alginic acid or alginates, polymannan or polyglycans, starch, natural gums. The semisynthetic polymers for example can be chosen from the group consisting of crosslinked collagen with agents such as aldehydes or precursors of the same, dicarboxylic acids or their halogenides, diamines, derivatives of

cellulose, hyaluronic acid, chitin or chitosan, gellan, xanthan, pectin or pectic acid, polyglycans, polymannan, agar, agarose, natural gum and glycosaminoglycans. Lastly, examples of synthetic polymers that can be used are polylactic acid, polyglycolic acid or copolymers of the same or their derivatives, polydioxanes, polyphosphazenes, polysulphonic resins, polyurethanes, PTFE.

The biomaterials thus obtained can be used in the cardiovascular field or in all applications involving contact with the blood or with highly vascularized body tissues where the biomaterial used needs to be totally free of thrombogenicity, besides having characteristics of biocompatibility and biodegradability due to the use of the ester derivatives of hyaluronic acid.

The above noted biomaterials can be used to advantage in various fields of surgery: in internal surgery, osteoarticular surgery, surgery to the nerves, anastomosis, viscoelastic, ophthalmic, oncological, plastic, otorhinolaryngological, abdominal-pelvic, urogynaecological and cardiovascular surgery, such as in the preparation of cardiac valves, vascular stents, in the prevention of post-surgical adhesions, in the prevention of hypertrophic scarring.

Moreover, the sulphated compounds associated with fibrin, and possibly with other biologically active substances, can be used for the preparation of surgical glues.

The biomaterials according to the present invention can be used in other fields besides the surgical field, for instance in haemodialysis, cardiology, dermatology, ophthalmology, otorhinolaryngology, dentistry, gynaecology, urology, in extracorporeal blood circulation and oxygenation and in cosmetics.

The above noted biomaterials in their various forms can also be used to advantage as supports for cell cultures such as mesenchymal

cells or mature cells to obtain connective tissue, glandular tissue and nerve tissue.

The instant biopolymers can also be used in processes to coat objects used both in the medical field and in other industrial sectors, to  
5 thereby give new biological characteristics to the surfaces of materials used as supports.

Examples of objects which can be thus coated are catheters, guide channels, probes, cardiac valves, soft tissue replacements, replacements of an animal origin such as cardiac valves from pigs,  
10 artificial tendons, bone and cardiovascular replacements, contact lenses, blood oxygenators, artificial kidneys, hearts, pancreas and liver, blood bags, syringes, surgical instruments, filtration systems, laboratory instruments, culture containers and containers for the regeneration of cells and tissues, supports for peptides, proteins, antibodies.

One method of coating the surfaces of these objects is the Plasma  
15 Coating technique described in international patent application No. WO96/24392, and illustrated in more detail hereafter in a preparation example. The process for the preparation of the compounds of the present invention mainly consists of two steps, the first involving the  
20 controlled N-deacetylation of the natural polysaccharide, and the second involving the specific sulphation reaction of the primary hydroxy or free amino functions of glucosamine.

Fractions of hyaluronic acid from biological and fermentation sources, with a molecular weight of between 5,000 and 5,000,000 Da, preferably between 50,000 Da and 300,000 Da, are solubilized in  
25 hydrazine hydroxide with a purity of no less than 98%, in a concentration range of between 1 and 50 mg/ml, preferably between 5 and 25 mg/ml. This solution is then supplemented with hydrazine sulphate in a weight/volume concentration varying between 0.1 and 3%,  
30 preferably 1%.

The reaction is conducted within a temperature range of 40 to 90°C, preferably 60°C, under agitation, for as long as it takes to reach the desired degree of N-deacetylation.

Table 1 hereafter reports the yield expressed as the percentage of free amino groups, in terms of time expressed as hours of reaction:

Table 1

Test	Temperature	Time (hours)	N-deacetylation (%)*
DAc 1**	60°	4	3
DAc 2	60°	8	5
DAc 3	60°	16	9
DAc 4	60°	24	14
DAc 5	60°	48	23
DAc 6	60°	72	36

\* The percentage of N-deacetylation is determined according to the method of J. Riesenfeld (Analy. Bioch. 1990, vol. 188, pages 383-389).

\*\* "DAc" = N-deacetylation

The reaction is then stopped by precipitation with a polar solvent, preferably ethanol. The precipitate is partially vacuum-dried and treated with a solution of iodic acid with a molarity range of between 0.1 and 1M, preferably 0.5M, and lastly, with iodohydric acid at a concentration of 57% (w/v). The pH of the solution is maintained between 5 and 7 by adding a solution of sodium acetate (10% w/v).

The aqueous phase containing the modified polysaccharide is extracted by repeated treatments with diethylether and then, once the yellow color has completely disappeared, the solution is treated again with ethanol.

The precipitate which forms, after further drying at 40°C, is solubilized in water at a concentration of between 10 ng/ml and 40 ng/ml, preferably 25 ng/ml, and the solution is percolated through a column containing an ion exchange resin activated with a tetraalkyl-ammonium hydroxide, where the alkyl residue of the quaternary

ammonium is constituted by a chain of between 1 and 4 carbon atoms; tetrabutylammonium hydroxide is preferably used.

The percolated product, represented by the quaternary ammonium salt of the modified polysaccharide, is then freeze-dried.

## 5      **Preparation of a partially 2-N-sulphated derivative:**

### **Method A**

10      The quaternary ammonium salt, preferably of tetrabutylammonium, of the partially N-deacetylated polysaccharide, is solubilized in an apolar solvent such as dimethyl sulphoxide, dimethyl formamide, dimethyl acetamide, N-methyl-pyrrolidone, preferably dimethyl formamide (DMFA), at a concentration of between 5 and 50 mg/ml (preferably 25 mg/ml).

15      The organic solution is supplemented with another solution obtained by solubilizing the sulphating complex constituted by dimethylformamide sulphotrioxide (DMFA-SO<sub>3</sub>), in DMFA, at a concentration varying between 50 and 200 mg/ml and preferably 100 mg/ml. The quantity of complex to be used, expressed in moles of SO<sub>3</sub>, proves surprisingly to be equivalent to the moles of amino groups released by the N-deacetylation reaction.

20      The sulphation reaction proceeds at a temperature of between 0° and 20°C, preferably 4°C for no longer than 4 hours and is then stopped by adding cold, distilled water.

25      The reaction solvent is first purified by precipitating the partially 2-N-sulphated hyaluronic acid with ethanol and then dialysing the resolubilized product with distilled water.

Lastly, the solution is freeze-dried and the solid product thus obtained undergoes chemical-analytical characterization to determine the degree of N-sulphation and the mean molecular weight (Table 2).

Table 2

Test	% deacetylation	% N-sulphation	mean MW (Da)
HA	0	0	165,000
HA-N-S1	5.0 (DAc 2)	4.8	157,000
HA-N-S2	14.2 (DAc 4)	13.9	147,000
HA-N-S3	23.5 (DAc 5)	23.0	139,000
HA-N-S4	36.1 (DAc 6)	34.2	124,000

HA = hyaluronic acid

HA-N-S = N-sulphated hyaluronic acid

## 5 Preparation of a partially 2-N-sulphated, 6-O-sulphated derivative:

### Method B

The quaternary ammonium salt, preferably of tetrabutyl-ammonium, of the partially N-deacetylated polysaccharide is solubilized in an apolar solvent such as dimethylsulphoxide, demethylformamide, dimethylacetamide, N-methyl-pyrrolidone, preferably dimethylform-  
 10 amide (DMFA), at a concentration of between 5 and 50 mg/ml, preferably 30 mg/ml.

The organic solution is supplemented with another solution obtained by solubilizing the sulphating complex constituted by dimethylformamide sulphotrioxide (DMFA-SO<sub>3</sub>), in DMFA, at con-  
 15 centrations varying between 50 and 200 mg/ml and preferably 100 mg/ml. The quantity of complex used, expressed as moles of SO<sub>3</sub>, prove surprisingly to be equivalent to the moles of amino groups released by the N-deacetylation reaction.

20 The sulphation reaction proceeds at a temperature of between 0° and 20°C, preferably at 4°C for 4 hours. A solution prepared by solubilizing the pyridine-sulphotrioxide complex in dimethylsulphoxide in such a quantity that the ratio between the moles of SO<sub>3</sub> of the



sulphating agent and the moles of  $\text{-CH}_2\text{OH}$  comes between 1.1 and 1.3. Larger quantities of reagent may favor any substitution reactions in other alcohol groups (secondary) of the polysaccharide chain.

The reaction then proceeds for another 16 hours at least after which it is stopped by adding cold, distilled water.

All subsequent steps concerning the purification of the modified polysaccharide are those described in "method A".

The analytical characterization performed on the derivatives obtained confirmed that the sulphation method proves surprisingly not only to substitute all the amino groups obtained by the partial N-deacetylation, but also results in the complete substitution of the primary alcohol group of the glucosamine residue of hyaluronic acid (Table 3).

Table 3

Test	% N-deacetylation	% N-sulphation	% 6-O-sulphation
HA-N-O-S1	5.0 (DAc 2)	4.8	100
HA-N-O-S1	14.2 (DAc 4)	13.9	99.2
HA-N-O-S1	23.5 (DAc 5)	23.0	98.9
HA-N-O-S1	36.1 (DAc 6)	34.2	96.5

HA-N-O-S1 = hyaluronic acid, N-sulphated and totally O-sulphated in position 6

Moreover, by varying the molar quantities of the pyridine- $\text{SO}_3$  complex according to the primary hydroxyl groups (molar ratio of between 0.1 and 1), "method B" enables a series of partially 2-N-sulphated and partially 6-O-sulphated derivatives to be obtained.

### Biological activity

The compounds prepared as described previously are characterized from a biological point of view in such a way as to

determine their anticoagulant activity. As reference products, we used the sulphated derivatives of hyaluronic acid obtained according to the method described in international patent application No. WP 95/25751, where the primary and secondary hydroxy groups of the polymer chain are substituted with  $-SO_3^-$  groups according to the quantity of sulphating agent that is used. Moreover, unfractionated heparin (UF heparin) is used as a further reference product, the efficacy of which as an anticoagulant drug has been widely acknowledged for many decades.

Subsequently, the thrombin time (TT test) and whole blood coagulation time (WBCT) were assessed for the following compounds:

Compound	Description
HA 200kDa	hyaluronic acid, sodium salt
UF Heparin	unfractionated heparin with mean MW = 15 kDa
HA-NS	partially (25%) 2-N-sulphated hyaluronic acid
HA-NS-OS	partially 2-N-sulphated (24%) and 6-O-sulphated hyaluronic acid
HA-OS	6-O-sulphated hyaluronic acid
HA-2S	hyaluronic acid sulphated with grade 2 substitution
HA-3S	hyaluronic acid sulphated with grade 3 substitution
HA-4S	hyaluronic acid sulphated with grade 4 substitution

The ability of the N-sulphated derivatives to prolong blood coagulation time is measured by the thrombin time test performed with a coagulometer. The time it takes to transform fibrinogen into fibrin after the addition of thrombin to a blood sample is determined in the presence of the polymer used as starting material. The test loses its significance when the time exceeds 120 seconds. The coagulation time is determined by simply observing the time it takes for a sample of human blood to coagulate in the presence of the test material. Any times exceeding two hours are not considered.

The results of the tests are reported in Table 4 hereafter:

Table 4

Compound	WBCT (sec)	TT (sec)	UF heparin equivalents <sup>a</sup>
Control	25	10	--
HA 200 kDa	45	11	--
HA-NS	>120	>120	0.46
HA-NS-OS	>120	>120	0.46
HA-OS	40	1	--
HA-2S	45	12.5	--
HA-3S	>120	38	0.013
HA-4S	>120	>120	0.46

<sup>a</sup>: mg of UF heparin necessary to obtain the same effect on coagulation time

Surprisingly, it seems clear that the chemical modification involving the amino group of the glucosamine residue of the polysaccharide chain induces a notable effect on the coagulation time. An identical result can only be obtained by drastically modifying the chemical structure of HA, that is, by substituting all the hydroxyls available per monomeric unit (four) with as many sulphonic groups.

The data obtained show that a degree of sulphation of 0.25, deriving from the previous N-deacetylation and subsequent N-sulphation, is sufficient to act specifically with the factors involving the terminal part of the coagulation cascade, thereby inhibiting the process of transformation of the fibrinogen into fibrin (anticoagulant activity). The presence of the sulphur group in position 6-O of the glucosamine residue does not seem to be a determining factor in the performance of this activity. However, in association with 2-N-sulphated groups, it may play a fundamental role in the inhibition of the Xa factor by interaction with ATIII (antithrombotic activity) and in the inhibition of the factor VIIIa (or vWf) in regulating platelet activation and proliferation.

### Analytical characterization

The chemical-analytical profiles of all the derivatives prepared and described in the present invention have been characterized. The following analytical methodologies were used:

5        Degree of sulphation (% of sulphur): after undergoing complete combustion in an oxygen-rich environment, the product was analyzed by HPLC using an ionic chromatography technique.

10        Percentage of 2-N-sulphation: this parameter is indirectly determined by measuring the total sulphonic groups present both before and after N-desulphation of the product, using the method described by Nagasawa (Carbohy. Res. 1977, 58, pages 47-55). The difference between the two values represents the quantity of  $\text{SO}_3^-$  groups linked to the amino group of glucosamine.

15        Percentage of N-deacetylation: this parameter is determined by the method described by J. Riesenfeld (Analy. Bioch. 1990, vol. 188, pages 383-389).

20        Mean molecular weight: this parameter is determined by GPC, using a set of Shadex and B-803 and B-806 columns, a multi-angle-laserlight-scattering monitor (MALLS) and a refractometer to measure the index of refraction (RI).

### EXAMPLES

#### Example 1

Preparation of partially 2-N-sulphated hyaluronic acid (wherein about 5% of the N-acetyl groups are substituted by sulphated groups)

25        1.00 gr of HA from rooster combs, with a mean molecular weight of 181,000 Da, is solubilized in 50 ml of hydrazine monohydrate together with 0.5 gr of hydrazine sulphate.

The solution is maintained under agitation while the reaction is continued for 8 hours at 60°C, after which it is stopped by the addition

of 100 ml of ethanol. The gelatinous precipitate thus formed is washed with ethanol and then dried at room temperature under reduced pressure.

5 The intermediate product is solubilized in a mixture constituted by 50 ml of water and 20 ml of a 10% solution of sodium acetate, and is treated lastly with 25 ml of a solution of iodic acid at a concentration of 0.5M. After about 30 minutes' reaction under agitation, the excess iodine is titrated with 5 ml of a 57% solution of iodohydric acid. During  
10 this operation it is preferable to keep the reaction container cold with ice. The rich brown solution is then treated at least five times with 30 ml aliquots of diethyl ether to extract the reaction residues from the aqueous solution containing the modified polymer. It is finally concentrated, at reduced pressure and at a temperature of 40°C, to a volume of about 40 ml and then percolated through a column filled with  
15 20 ml of ion exchange sulphonic resin activated with a 40% solution w/v of tetrabutylammonium hydroxide.

The aqueous solution containing the modified polysaccharide in the form of tetrabutylammonium salt (HATBA) is then harvested and subjected to one lyophilization cycle.

20 1.30 gr of freeze-dried HA salt of TBA is solubilized in 45 ml of dimethylformamide and the solution thus obtained is supplemented with 0.6 ml of a solution of a complex of N-N dimethylformamide sulphotrioxide at a concentration of 50 mg/ml. The reaction continues for 5 hours at 4°C under continuous, gentle agitation, after which it is stopped  
25 by adding 45 ml of cold, distilled water. Having neutralized the solution with NaOH 2 M, bringing it to a pH of between 7.5 and 8, it is then filtered through a Gooch filter with pore size G2 and treated with 250 ml of ethanol.

30 The precipitate thus formed is washed with at least 150 ml of ethanol and vacuum-dried for at least 16 hours, after which it is

resolubilized in 50 ml of distilled water and then dialysed against 50 volumes of water.

The product is freeze-dried and then characterized to determine the percentage of N-substituted amino groups and its mean molecular weight.

Weight of the freeze-dried product:	0.72 gr; yield: 85%
moles of $\text{SO}_3^-$ /moles of HA (monomeric units)	0.045
moles of free $\text{-NH}_2$ groups/moles of HA:	0.052
% of de-N-acetylation:	5.2%
% of re-N-sulphation	4.5%
yield from the N-sulphation reaction:	87%
mean molecular weight:	174,000 Da

## Example 2

### Preparation of partially 2-N-sulphated hyaluronic acid (wherein about 25% of the N-acetyl groups are substituted with sulphated groups)

1.2 gr of HA from rooster combs, with a mean molecular weight of 181,000 Da, is solubilized in 60 ml of hydrazine monohydrate together with 0.6 gr of hydrazine sulphate.

The solution is maintained under agitation while the reaction proceeds for 24 hours at 60°C, after which it is stopped by the addition of 120 ml of ethanol. The gelatinous precipitate thus formed is washed with ethanol and then dried at room temperature under reduced pressure.

The intermediate product is solubilized in a mixture constituted by 60 ml of water and 25 ml of a 10% solution of sodium acetate, and is treated lastly with 30 ml of a solution of iodic acid at a concentration of 0.5M. After about 30 minutes' reaction under continuous agitation, the excess iodine is titrated with 6 ml of a 57% solution of iodohydric acid.

During this operation it is preferable to keep the reaction container cold with ice.

The rich brown solution is then treated at least five times with 40 ml aliquots of diethyl ether to extract the reaction residues from the aqueous solution containing the modified polymer. It is finally concentrated, at reduced pressure and at a temperature of 40°C, to a volume of about 50 ml and then percolated through an ion exchange column filled with 25 ml of sulphonic resin activated with a 40% solution w/v of tetrabutylammonium hydroxide.

The aqueous solution containing the modified polysaccharide in the form of tetrabutylammonium salt (HATBA) is then harvested and subjected to one lyophilization cycle.

1.65 gr of freeze-dried HA salt of TBA is solubilized in 55 ml of dimethylformamide and the solution thus obtained is supplemented with 3.0 ml of solution at a concentration of 50 mg/ml of a complex of N-N dimethylformamide sulphotrioxide. The reaction continues for 6 hours at 4°C under continuous, gentle agitation, after which it is stopped by adding 55 ml of cold, distilled water. Having neutralized the solution with NaOH 2 M, bringing it to a pH of between 7.5 and 8, it is then filtered through a Gooch filter with pore size G2 and treated with 300 ml of ethanol.

The precipitate thus formed is washed with at least 150 ml of ethanol and vacuum-dried for at least 16 hours, after which it is resolubilized in 50 ml of distilled water and then dialysed against 50 volumes of water.

The product is freeze-dried and then characterized to determine the percentage of N-substituted amino groups and its mean molecular weight.

Weight of the freeze-dried product:	0.98 gr; yield: 89%
moles of SO <sub>3</sub> <sup>-</sup> /moles of HA (monomeric units)	0.23

	moles of free -NH <sub>2</sub> groups/moles of HA:	0.24
	% of de-N-acetylation:	24%
	% of re-N-sulphation:	23%
	yield from the N-sulphation reaction:	96%
5	mean molecular weight:	161,000 Da

### Example 3

Preparation of hyaluronic acid, partially 2-N-sulphated (wherein about 25% of the N-acetyl groups are substituted by sulphated groups) and 6-O-sulphated

10        5.0 gr of HA obtained by fermentation, with a mean molecular weight of 195,000 Da, is solubilized in 250 ml of hydrazine monohydrate together with 2.5 gr of hydrazine sulphate.

15        The reaction is maintained under agitation for 24 hours at 60°C, after which it is stopped by the addition of 500 ml of ethanol. The gelatinous precipitate thus formed is washed with ethanol and then dried at room temperature under reduced pressure.

20        The intermediate product is solubilized in a mixture constituted by 250 ml of water and 105 ml of a 10% solution of sodium acetate, and is treated lastly with 125 ml of a solution of iodic acid at a concentration of 0.5M. After about 30 minutes' reaction under continuous agitation, the excess iodine is titrated with 25 ml of a 57% solution of iodohydric acid. During this operation it is preferable to keep the reaction container cold with ice.

25        The rich brown solution is then treated at least five times with 150 ml aliquots of diethyl ether to extract the reaction residues from the aqueous solution containing the modified polymer. It is finally concentrated, at reduced pressure and at a temperature of 40°C, to a volume of about 200 ml and then percolated through a column filled



with 100 ml of ion exchange sulphonic resin activated with a 40% solution w/v of tetrabutylammonium hydroxide.

The aqueous solution containing the modified polysaccharide in the form of tetrabutylammonium salt (HATBA) is then harvested and subjected to one lyophilization cycle.

6.0 gr of freeze-dried HA salt of TBA is solubilized in 300 ml of dimethylformamide and the solution thus obtained is supplemented with 13 ml of a solution of a complex of N-N dimethylformamide sulphotrioxide at a concentration of 50 mg/ml. The reaction continues for 6 hours at 4°C under continuous, gentle agitation.

A second solution, constituted by 40 ml of a complex of pyridine sulphotrioxide solubilized in dimethyl sulphoxide at a concentration of 50 mg/ml is added to the reaction mixture.

Approximately sixteen hours later, 250 ml of cold, distilled water is added and, once the solution has been neutralized with NaOH 2 M to a pH of 8, it is then filtered through a Gooch filter with pore size G3 and treated with 1,250 ml of ethanol.

The precipitate thus formed is washed with at least 500 ml of ethanol and vacuum-dried for at least 16 hours, after which it is resolubilized in 250 ml of distilled water and then dialyzed against 50 volumes of water.

The product is freeze-dried and then characterized to determine the percentage of N-substituted amino groups, the degree of 6-O-sulphation and its mean molecular weight.

Weight of the freeze-dried product:	4.12 gr; yield: 82%
moles of SO <sub>3</sub> -/moles of HA (monomeric units)	1.24
moles of free -NH <sub>2</sub> groups/moles of HA:	0.26
% of de-N-acetylation:	26%
% of re-N-sulphation:	24%
% of O-sulphation:	100%

mean molecular weight: 170,000 Da

#### Example 4

##### Preparation of the benzyl ester of hyaluronic acid, partially N-sulphated and O-sulphated

5           2.00 gr of the derivative obtained in Example 3 is solubilized in 100 ml of distilled water and the solution is percolated through a glass column previously filled with 40 ml of ion exchange resin activated with tetrabutylammonium hydroxide (TBA<sup>+</sup> form). The eluate is freeze-dried and 3.3 gr of product is obtained.

10           The product is solubilized in a mixture constituted by 130 ml of N-methyl pyrrolidone and 1.3 ml of water, reacted at 4°C with 0.29 ml of benzyl bromide. The reaction proceeds for 48 hours at 28°C, keeping the solution under agitation and away from sources of light, after which 300 ml of ethyl acetate is added.

15           The precipitate thus formed, mainly constituted by the modified polysaccharide, is washed with 100 ml of acetone and then vacuum-dried at room temperature, after which it is treated with 100 ml of a 10% solution w/v of sodium chloride.

20           At the end of the saline treatment (which lasts about one hour), the product is washed with 150 ml of water/acetone 20:80 and lastly with 100 ml of acetone.

          After drying for 48 hours at 30°C, 0.92 gr at a yield of 80% is obtained.

#### Characterization:

25           % of esterification 96%

**Example 5**Preparation of 10% autocrosslinked hyaluronic acid, partially N-sulphated and O-sulphated

2.00 gr of the derivative obtained in example 3 are solubilized in 100 ml of distilled water and the solution is percolated through a glass column filled with 40 ml of ion exchange resin activated with tetrabutylammonium hydroxide (TBA<sup>+</sup> form). After freeze-drying the eluate, 3.3 gr of product are obtained.

The product is solubilized in a mixture formed by 165 ml of N-methyl pyrrolidone (NMP) and 0.8 ml of water, and then reacted with a solution obtained by solubilizing 205 mg of 2-chloro-1-methyl pyridine iodide in 8.2 ml of NMP. The reaction proceeds for 18 hours at -20°C, after which 165 ml of an aqueous solution of 3% ammonium acetate is added.

The mixture is constantly agitated for about 4 hours and then treated with 650 ml of ethanol. The precipitate thus formed is separated by filtration, washed with ethanol and then vacuum-dried for 24 hours.

The product is then treated with 60 ml of a 3% solution of sodium chloride so as to favor ion exchange and lastly reprecipitated by adding 180 ml of ethanol to the solution. After eliminating the supernatant the product is washed at least three times with 50 ml of ethanol and is then treated with 100 ml of acetone before being finally dried at 30°C for 48 hours.

0.97 gr of sulphated and partially autocrosslinked derivative are thus obtained.

**Example 6**Preparation of a film of benzyl ester of hyaluronic acid partially N-sulphated and O-sulphated

A solution of the benzyl ester of hyaluronic acid, partially N-sulphated and O-sulphated is prepared in dimethylsulphoxide at a concentration of 180 mg/ml.

5 A thin layer of solution is spread over a glass plate; the thickness of the layer of solution must be 10 times greater than that of the final film. The glass plate is immersed in ethanol which absorbs the dimethylsulphoxide without solubilizing the ester, which solidifies. The film is separated from the glass plate and repeatedly washed with ethanol, water and then again with ethanol.

10 The film obtained is dried under pressure for 48 hours at 30°C.

### Example 7

Preparation of the silver salt of the partially 2-N-sulphated (25%) and 6-O-sulphated hyaluronic acid derivative

15 0.50 gr of compound obtained according to example 2, is solubilized in 25 ml of distilled water and the solution obtained is percolated through a column filled with 16 cm<sup>3</sup> of strong ion exchange resin in H<sup>+</sup> form. The eluate is then harvested and freeze-dried. The intermediate product in acid form obtained by freeze-drying is treated with 20 ml of a 0.5 M solution of AgNO<sub>3</sub> for 60 minutes under agitation and away from the light.

20 Having eliminated the liquid phase by filtration, the product is thoroughly washed with 150 ml of distilled water and then with 50 ml of absolute ethanol. After vacuum-drying the sulphated hyaluronic acid derivative, silver salt, at 40°C, 0.649 gr are obtained (yield 95%).

### 25 Example 8

Coating of a cardiac valve made of polyurethane with partially 2-N-sulphated (25%) and 6-O-sulphated hyaluronic acid

A cardiac valve made of polyurethane is treated with oxygen plasma produced with a radio frequency generator.

The working conditions are as follows: the pressure in the reaction chamber is set at 100 mtorr, the strength of the plasma generator is 50 W, the oxygen flow is set at 20 cm<sup>3</sup>/min. and the treatment time is 30 sec.

The device thus treated is then immersed in 250 ml of a 0.65% solution of polyethylene amine with a molecular weight of 500,000, and left to soak there for 90 minutes. After thorough washing with distilled water, the material is placed in contact with 250 ml of an aqueous solution obtained by solubilizing 2.5 gr of partially 2-N-sulphated and 6-O-sulphated derivative, obtained as described in example 2. Moreover, the following substances are added in stoichiometric quantities with regard to the carboxy groups belonging to the modified polysaccharide: 0.76 gr of N-hydroxysuccinimide (NHS) and 1.23 gr of 3-dimethylaminopropyl-1-ethyl carbodiimide (EDC). The reaction proceeds for about 16 hours at room temperature.

Lastly, the device coated with the sulphated hyaluronic acid derivative is thoroughly washed with distilled water and then blown dry.

The invention being thus described, it is clear that these methods can be modified in various ways. Such modifications are not to be considered as divergences from the spirit and purpose of the invention and any such modifications as would appear evident to an expert in the field come within the scope of the following claims.

## CLAIMS

1. A sulphated hyaluronic acid compound, a derivative thereof, or a salt thereof, wherein the glucosamines of said compound or said derivative thereof are partially N-sulphated or partially N-sulphated and totally or partially O-sulphated in position 6.

2. The sulphated hyaluronic acid compound according to claim 1, wherein said hyaluronic acid derivative is a total or partial ester with an aliphatic, aromatic, arylaliphatic, cycloaliphatic or heteroaliphatic alcohol.

3. The sulphated hyaluronic acid compound according to claim 2, wherein said hyaluronic acid derivative is selected from the group consisting of:

a total ester of hyaluronic acid with benzyl alcohol,

a partial ester of hyaluronic acid wherein about 25% of the carboxy groups of the hyaluronic acid are esterified with benzyl alcohol,

a partial ester of hyaluronic acid wherein about 50% of the carboxy groups of the hyaluronic acid are esterified with benzyl alcohol,

a partial ester of hyaluronic acid wherein about 75% of the carboxy groups of the hyaluronic acid are esterified with benzyl alcohol,

a total ester of hyaluronic acid with ethyl alcohol,

a partial ester of hyaluronic acid wherein about 25% of the carboxy groups of the hyaluronic acid are esterified with ethyl alcohol,

a partial ester of hyaluronic acid wherein about 50% of the carboxy groups of the hyaluronic acid are esterified with ethyl alcohol,

a partial ester of hyaluronic acid wherein about 75% of the carboxy groups of the hyaluronic acid are esterified with ethyl alcohol,

an ester of hyaluronic acid wherein the carboxy groups of the hyaluronic acid are esterified with dodecyl alcohol to an extent of between 5% and 100%, and

an ester of hyaluronic acid wherein the carboxy groups of the hyaluronic acid are esterified with hexadecyl alcohol to an extent of between 5% and 100%.

4. The sulphated hyaluronic acid compound according to claim 1, wherein said hyaluronic acid derivative is a crosslinked compound, wherein a part or all the carboxy groups of the D-glucuronic residue form inner esters or inter-molecular esters with the alcohol functions of the same polysaccharide chain or other chains respectively.

5. The sulphated hyaluronic acid compound according to claim 1, wherein the hyaluronic acid derivative is a crosslinked compound, wherein said crosslinked compound is formed by reacting a part or all of the carboxy groups of the D-glucuronic residue with polyalcohols of an aliphatic, aromatic, arylaliphatic, cycloaliphatic or heterocyclic series, to thereby generate crosslinking by means of spacer chains.

6. The sulphated hyaluronic acid compound according to claim 1, wherein said compound or said derivative thereof is salified with a heavy metal.

7. The sulphated hyaluronic acid compound according to claim 6, wherein the heavy metal is a metal element selected from the 4th, 5th and 6th groups of the periodic table of elements.

8. The sulphated hyaluronic acid compound according to claim 7, wherein said heavy metal is silver, cobalt, iron, copper, zinc, arsenic, strontium, zirconium, antimony, gold, cesium, tungsten, selenium, platinum, ruthenium, bismuth, tin, titanium, or mercury.

9. The sulphated hyaluronic acid compound according to claim 1, wherein said compound or said derivative thereof that is salified with a pharmacologically active substance.

10. The sulphated hyaluronic acid compound according to claim 9, wherein the pharmacologically active substance is selected from the group consisting of an antibiotic, an antiinfective, an antimicrobial, an antiviral, a cytostatic, an antitumoral, an antiinflammatory, a wound healing agent, an anaesthetic, a cholinergic agonist, a cholinergic antagonist, an adrenergic agonist, an adrenergic antagonist, an antithrombotic, an anticoagulant, a haemostatic, a fibrinolytic, a thrombolytic agent, a protein, a protein fragment, a peptide, and a polynucleotide.

11. The sulphated hyaluronic acid compound according to claim 1, wherein the degree of sulphation per a dimeric unit of the amino groups varies between 1 and 70% and that of the hydroxyl group in position 6 varies between 0 and 100%.

12. The sulphated hyaluronic acid compound according to claim 1, wherein the degree of sulphation per a dimeric unit of the amino groups varies between 5 and 40% and that of the hydroxyl group in position 6 varies between 0 and 100%.



13. A pharmaceutical composition containing therein a pharmaceutically effective amount of a sulphated hyaluronic acid compound or a derivative thereof, wherein the glucosamines of said compound or said derivative thereof are partially N-sulphated or partially N-sulfated and totally or partially O-sulphated in position 6, and said compound or said derivative optionally being salified and optionally being in association with another pharmacologically active substance, and a pharmaceutically acceptable carrier therefor.

14. The pharmaceutical composition according to claim 13, wherein said hyaluronic acid derivative is a total or partial ester with an aliphatic, aromatic, arylaliphatic, cycloaliphatic or heteroaliphatic alcohol.

15. The pharmaceutical composition according to claim 14, wherein said hyaluronic acid derivative is selected from the group consisting of:

- a total ester of hyaluronic acid with benzyl alcohol,
- a partial ester of hyaluronic acid wherein about 25% of the carboxy groups of the hyaluronic acid are esterified with benzyl alcohol,
- a partial ester of hyaluronic acid wherein about 50% of the carboxy groups of the hyaluronic acid are esterified with benzyl alcohol,
- a partial ester of hyaluronic acid wherein about 75% of the carboxy groups of the hyaluronic acid are esterified with benzyl alcohol,
- a total ester of hyaluronic acid with ethyl alcohol,
- a partial ester of hyaluronic acid wherein about 25% of the carboxy groups of the hyaluronic acid are esterified with ethyl alcohol,
- a partial ester of hyaluronic acid wherein about 50% of the carboxy groups of the hyaluronic acid are esterified with ethyl alcohol,

a partial ester of hyaluronic acid wherein about 75% of the carboxy groups of the hyaluronic acid are esterified with ethyl alcohol,

an ester of hyaluronic acid wherein the carboxy groups of the hyaluronic acid are esterified with dodecyl alcohol to an extent of between 5% and 100%, and

an ester of hyaluronic acid wherein the carboxy groups of the hyaluronic acid are esterified with hexadecyl alcohol to an extent of between 5% and 100%.

16. The pharmaceutical composition according to claim 13, wherein said hyaluronic acid derivative is a crosslinked compound, wherein a part or all the carboxy groups of the D-glucuronic residue form inner esters or inter-molecular esters with the alcohol functions of the same polysaccharide chain or other chains respectively.

17. The pharmaceutical composition according to claim 13, wherein the hyaluronic acid derivative is a crosslinked compound, wherein said crosslinked compound is formed by reacting a part or all of the carboxy groups of the D-glucuronic residue with polyalcohols of an aliphatic, aromatic, arylaliphatic, cycloaliphatic or heterocyclic series, to thereby generate crosslinking by means of spacer chains.

18. The pharmaceutical composition according to claim 13, wherein said compound or said derivative thereof is salified with a heavy metal.

19. The pharmaceutical composition according to claim 13, wherein the heavy metal is a metal element selected from the 4th, 5th and 6th groups of the periodic table of elements.

20. The pharmaceutical composition according to claim 13, wherein said heavy metal is silver, cobalt, iron, copper, zinc, arsenic, strontium, zirconium, antimony, gold, cesium, tungsten, selenium, platinum, ruthenium, bismuth, tin, titanium, or mercury.

21. The pharmaceutical composition according to claim 13, wherein said compound or said derivative thereof is salified with a pharmacologically active substance.

22. The pharmaceutical composition according to claim 13, wherein the pharmacologically active substance is selected from the group consisting of an antibiotic, an antiinfective, an antimicrobial, an antiviral, a cytostatic, an antitumoral, an antiinflammatory, a wound healing agent, an anaesthetic, a cholinergic agonist, a cholinergic antagonist, an adrenergic agonist, an adrenergic antagonist, an antithrombotic, an anticoagulant, a haemostatic, a fibrinolytic, a thrombolytic agent, a protein, a protein fragment, a peptide, a polynucleotide, growth factors, enzymes, vaccines, substances used in the treatment of diseases associated with genetic defects, deforming disorders and hereditary diseases.

23. The pharmaceutical composition according to claim 13, wherein the degree of sulphation per a dimeric unit of the amino groups varies between 1 and 70% and that of the hydroxyl group in position 6 varies between 0 and 100%.

24. The pharmaceutical composition according to claim 13, wherein the degree of sulphation per a dimeric unit of the amino groups varies between 5 and 40% and that of the hydroxyl group in position 6 varies between 0 and 100%.

25. A biomaterial comprising a sulphated hyaluronic acid compound or a derivative thereof wherein the glucosamines of said compound or said derivative thereof are partially N-sulphated or partially N-sulfated and totally or partially O-sulphated in position 6, and said composition or said derivative optionally being salified and optionally being in association with a natural, a semisynthetic or a synthetic polymer, and, optionally further being in combination with a biologically active substance.

26. A biomaterial according to claim 25, wherein the natural polymer is selected from the group consisting of a collagen, a coprecipitate of collagen and glycosaminoglycan, a cellulose, a polysaccharides in the form of a gel selected from the group consisting of chitin, chitosan, pectin, pectic acid, agar, agarose, xanthan, gellan, alginic acid, an alginate, polymannan, a polyglycan, starch, and a natural gum.

27. A biomaterial according to claim 25, wherein the semisynthetic polymer is a collagen crosslinked with a crosslinking agent selected from the group consisting of an aldehyde, a precursor of an aldehyde, a dicarboxylic acid, a dicarboxylic acid halogenide, a diamine, a cellulose derivative, hyaluronic acid, chitin, chitosan, gellan, xanthan, pectin, pectic acid, a polyglycan, polymannan, agar, agarose, natural gum and glycosaminoglycans.

28. A biomaterial according to claim 25, wherein the synthetic polymer is selected from the group consisting of polylactic acid, polyglycolic acid, a copolymer of polylactic acid, a derivative of polylactic acid, a copolymer of polyglycolic acid, a derivative of

polyglycolic acid, polydioxan, polyphosphazene, a polysulphonic resin, polyurethane and PTFE.

29. A biomaterial according to claim 25, in association with fibrin, and optionally with another biologically active substance, and which biomaterial is a surgical glue.

30. A biomaterial according to claim 25, which is a support for cell cultures.

31. A biomaterial according to claim 25, which is selected from the group consisting of a guide channel, a thread, a gel, a hydrogel, a buffer, a film, a membrane, a sponge, a nonwoven fabric, microspheres and nanospheres.

32. A biomaterial according to claim 25, which is a health care or surgical article.

33. A biomaterial according to claim 32, which said health care or surgical article is selected from the group consisting of a guide channel, a gauze, a thread, a gel, a hydrogel, a buffer, a film, a membrane, a sponge, a nonwoven fabric, microspheres and nanospheres.

34. Use of a biomaterial according to claim 25 in surgery, haemodialysis, cardiology, dermatology, ophthalmology, otorhinolaryngology, dentistry, gynaecology, urology, in extracorporeal blood circulation and oxygenation and in cosmetics.

35. Use of a biomaterial according to claim 34, wherein said surgery is selected from the group consisting of internal, osteoarticular surgery, surgical anastomosis, neurosurgery, viscoelastic, ophthalmic, oncological, plastic, otorhinolaryngological, abdominal-pelvic, urogynaecological and cardiovascular surgery.

36. Use of the biomaterial according to claims 25 in association with fibrin and possibly other biologically active substances for the preparation of surgical glues.

37. Use of the biomaterial according to claim 25 for the preparation of supports for cell cultures.

38. A biomedical object coated with a sulphated hyaluronic acid compound or a derivative thereof, wherein the glucosamines of said compound or said derivative thereof are partially N-sulphated or partially N-sulfated and totally or partially O-sulphated in position 6, wherein said biomedical object is selected from the group consisting of a bypass, a venous catheter, a shunt, a catheter, a guide channel, a probe, cardiac valves, artificial tendons, bone and cardiovascular replacements, contact lenses, soft tissue replacements, replacements of animal origin, blood oxygenators, artificial kidneys, hearts, pancreas and livers, blood bags, syringes, surgical instruments, filtration systems, laboratory instruments, containers for cultures and for the regeneration of cells and tissues, supports for peptides, proteins and antibodies.

39. A process for the preparation of a sulphated hyaluronic acid compound or a derivative thereof, wherein the glucosamines of said compound or said derivative thereof are partially N-sulphated or

partially N-sulfated and totally or partially O-sulphated in position 6, comprising the following steps:

a) carrying out a controlled N-deacetylation in hydrazine monohydrate of hyaluronic acid or the derivative thereof by adding hydrazine sulphate to a solution of the starting product;

b) preparing a quaternary ammonium salt of the N-deacetylated compound;

c) N-sulphating the quaternary ammonium salt by supplementing a solution of the salt in an apolar solvent with a solution of the sulphating complex which is selective for the amino group; and

d) optionally, O-sulphating the hydroxyl at position 6 by adding to the mixture of solutions in c) another solution of a sulphating complex selective for the primary hydroxy group.

40. The process for the preparation of the sulphated hyaluronic acid compound according to claim 39, wherein the sulphating complex selective for the amino group is dimethylformamide sulphotrioxide.

41. The process for the preparation of the sulphated hyaluronic acid compound according to claim 39, wherein the solution of the sulphating complex selective for the hydroxy group is a solution of pyridine sulphotrioxide in dimethyl sulphoxide.

42. A sulphated hyaluronic acid compound, a derivative thereof, or a salt thereof according to claim 1, alone or in association with one another and/or with a pharmacologically active substance for the preparation of pharmaceutical compositions.

43. The sulphated compound according to claim 42, wherein the pharmacologically active substance is selected from the group

consisting of antibiotics, anti-infective, antimicrobial, antiviral, cytostatic, antitumoral, anti-inflammatory and wound healing agents, anaesthetics, cholinergic or adrenergic agonists and antagonists, antithrombotic, anticoagulant, haemostatic, fibrinolytic, thrombolytic agents, proteins and their fragments, peptides, polynucleotides, growth factors, enzymes, vaccines, substances used in the treatment of diseases associated with genetic defects, deforming disorders and hereditary diseases.

44. Use of the sulphated compound according to claim 42, in association with radioactive and non-radioactive substances, used in contrast systems, to be used as markers in *in vivo* diagnostics to identify or treat tumoral or damaged tissues.

45. Use of the biomaterial according to claim 25 in surgery, wherein the surgery is selected from the group consisting of internal, osteoarticular surgery, surgical anastomosis, neurosurgery, viscoelastic, ophthalmic, oncological, plastic, otorhinolaryngological, abdominal-pelvic, urogynaecological, cardiovascular surgery, such as in the preparation of cardiac valves, vascular stents, in the prevention of post-operative adhesions and in the prevention of hypertrophic scars.

46. Use of the sulfated compound, derivative thereof, or salt thereof according to claim 1, for the treatment of inflammation, as an antiviral agent and to accelerate wound healing of burns, sores and skin ulcers, and to favor angiogenesis.

47. Use of the sulfated compound, derivative thereof or salt thereof according to claim 1, for the treatment of viral diseases caused by HIV and herpes.



48. Use of hyaluronic acid, partially N-sulphated or partially N-sulphated and totally or partially O-sulphated in position 6, for the preparation of hyaluronic acid esters according to claim 2.

49. Use of hyaluronic acid, partially N-sulphated or partially N-sulphated and totally or partially O-sulphated in position 6, for the preparation of crosslinked hyaluronic acid according to claim 4.

50. Use of hyaluronic acid, partially N-sulphated or partially N-sulphated and totally or partially O-sulphated in position 6, for the preparation of crosslinked hyaluronic acid according to claim 5.



# INTERNATIONAL SEARCH REPORT

International Application No  
PCT/EP 98/01973

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C08B37/08 A61K31/725 A61L25/00 A61L15/00 A61L27/00  
A61L33/00

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C08B

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0 340 628 A (CRINOS INDUSTRIA FARMACOBIOLOGICA SPA) 8 November 1989 cited in the application see abstract; examples 6,7 ---	1,11-13, 23,24,42
A	WO 95 25751 A (FIDIA ADVANCED BIOPOLYMERS SRL) 28 September 1995 cited in the application ---	1-3, 11-15, 23-25, 31-35, 38,42, 45,46
A	EP 0 464 759 A (HOECHST AKTIENGESELLSCHAFT) 8 January 1992 see claims --- -/-	47

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

### \* Special categories of cited documents :

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
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Date of the actual completion of the international search

2 September 1998

Date of mailing of the international search report

21/09/1998

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2  
NL - 2280 HV Rijswijk  
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl.  
Fax: (+31-70) 340-3016

Authorized officer

Mazet, J-F

# INTERNATIONAL SEARCH REPORT

Intern      ial Application No

PCT/EP 98/01973

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No. .
A	<p>MAGNANI A. ET AL.: "Blood-interaction performance of differently sulfated hyaluronic acids"</p> <p>THROMBOSIS RESEARCH,</p> <p>vol. 81, no. 3, 1996, pages 383-395,</p> <p>XP002076200</p> <p style="text-align: center;">-----</p>	

# INTERNATIONAL SEARCH REPORT

Information on patent family members

Interns 31 Application No

PCT/EP 98/01973

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
- EP 340628 A	08-11-1989	AT 109163 T AU 618346 B AU 3392289 A CA 1307525 A DE 68917009 D DE 68917009 T ES 2057006 T JP 1318002 A PT 90414 A, B US 5008253 A	15-08-1994 19-12-1991 02-11-1989 15-09-1992 01-09-1994 10-11-1994 16-10-1994 22-12-1989 30-11-1989 16-04-1991
WO 9525751 A	28-09-1995	IT PD940054 A AU 2072795 A CA 2163337 A EP 0702699 A JP 9510493 T	25-09-1995 09-10-1995 28-09-1995 27-03-1996 21-10-1997
EP 464759 A	08-01-1992	DE 4021066 A AU 7947091 A CA 2046037 A JP 4230325 A PT 98185 A	09-01-1992 09-01-1992 04-01-1992 19-08-1992 28-02-1994



# PATENT COOPERATION TREATY

WO 98/45335  
PCT/EP98/01973

PCT

## NOTICE INFORMING THE APPLICANT OF THE COMMUNICATION OF THE INTERNATIONAL APPLICATION TO THE DESIGNATED OFFICES

(PCT Rule 47.1(c), first sentence)

From the INTERNATIONAL BUREAU

To:

VOSSIUS & PARTNER  
Siebertstrasse 4  
D-81675 München  
ALLEMAGNE

**EINGEGANGEN**  
Vossius & Partner GbR

26. Okt. 1998

Frist  
bearb.

Date of mailing (day/month/year)

15 October 1998 (15.10.98)

Applicant's or agent's file reference

C 1498 PCT

### IMPORTANT NOTICE

International application No.

PCT/EP98/01973

International filing date (day/month/year)

03 April 1998 (03.04.98)

Priority date (day/month/year)

04 April 1997 (04.04.97)

Applicant

FIDIA ADVANCED BIOPOLYMERS, S.R.L. et al

1. Notice is hereby given that the International Bureau has communicated, as provided in Article 20, the international application to the following designated Offices on the date indicated above as the date of mailing of this Notice:

AU,BR,CA,CN,EP,IL,JP,KP,KR,NO,PL,US

In accordance with Rule 47.1(c), third sentence, those Offices will accept the present Notice as conclusive evidence that the communication of the international application has duly taken place on the date of mailing indicated above and no copy of the international application is required to be furnished by the applicant to the designated Office(s).

2. The following designated Offices have waived the requirement for such a communication at this time:

AL,AM,AP,AT,AZ,BA,BB,BG,BY,CH,CU,CZ,DE,DK,EA,EE,ES,FI,GB,GE,GH,GM,GW,HU,ID,IS,KE,  
KG,KZ,LC,LK,LR,LS,LT,LU,LV,MD,MG,MK,MN,MW,MX,NZ,OA,PT,RO,RU,SD,SE,SG,SI,SK,SL,TJ,  
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The communication will be made to those Offices only upon their request. Furthermore, those Offices do not require the applicant to furnish a copy of the international application (Rule 49.1(a-bis)).

3. Enclosed with this Notice is a copy of the international application as published by the International Bureau on 15 October 1998 (15.10.98) under No. WO 98/45335

### REMINDER REGARDING CHAPTER II (Article 31(2)(a) and Rule 54.2)

If the applicant wishes to postpone entry into the national phase until 30 months (or later in some Offices) from the priority date, a demand for international preliminary examination must be filed with the competent International Preliminary Examining Authority before the expiration of 19 months from the priority date.

It is the applicant's sole responsibility to monitor the 19-month time limit.

Note that only an applicant who is a national or resident of a PCT Contracting State which is bound by Chapter II has the right to file a demand for international preliminary examination.

### REMINDER REGARDING ENTRY INTO THE NATIONAL PHASE (Article 22 or 39(1))

If the applicant wishes to proceed with the international application in the national phase, he must, within 20 months or 30 months, or later in some Offices, perform the acts referred to therein before each designated or elected Office.

For further important information on the time limits and acts to be performed for entering the national phase, see the Annex to Form PCT/IB/301 (Notification of Receipt of Record Copy) and Volume II of the PCT Applicant's Guide.

The International Bureau of WIPO  
34, chemin des Colombettes  
1211 Geneva 20, Switzerland

Facsimile No. (41-22) 740.14.35

Authorized officer

J. Zahra

Telephone No. (41-22) 338.83.38





Continuation of Form PCT/IB/308

NOTICE INFORMING THE APPLICANT OF THE COMMUNICATION OF  
THE INTERNATIONAL APPLICATION TO THE DESIGNATED OFFICES

<b>Date of mailing (day/month/year)</b> 15 October 1998 (15.10.98)	<b>IMPORTANT NOTICE</b>
<b>Applicant's or agent's file reference</b> C 1498 PCT	<b>International application No.</b> PCT/EP98/01973
<p>The applicant is hereby notified that, at the time of establishment of this Notice, the time limit under Rule 46.1 for making amendments under Article 19 has not yet expired and the International Bureau had received neither such amendments nor a declaration that the applicant does not wish to make amendments.</p>	

